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Molecular detection of *Fusarium solani* f. sp. *glycines* in soybean roots and soil

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A polymerase chain reaction (PCR)-based method was developed to detect DNA of *Fusarium solani* f. sp. *glycines*, the cause of soybean sudden death syndrome. Two pairs of primers, Fsg1/Fsg2 designed from the mitochondrial small subunit ribosomal RNA gene, and FsgEF1/FsgEF2 designed from the translation elongation factor 1-α gene, produced PCR products of 438 and 237 bp, respectively. Primer specificity was tested with DNA from 82 *F. solani* f. sp. *glycines*, 55 *F. solani* non-SDS isolates, 43 isolates of 17 soybean fungal pathogens and the oomycete *Phytophthora sojae*, and soybean. The sensitivity of primer Fsg1/Fsg2 was 10 pg while that of FsgEF1/FsgEF2 was 1 ng when using *F. solani* f. sp. *glycines* total genomic DNA or down to 10^3 macroconidia g^{-1} soil. Nested PCR increased the sensitivity of the PCR assay 1000-fold to 10 fg using primers Fsg1/Fsg2, and 1 pg using primers FsgEF1/FsgEF2. *F. solani* f. sp. *glycines* DNA was detected in field-grown soybean roots and soil by PCR using either single pairs of primers or the combination of two pairs of primers. The occurrence of *F. solani* f. sp. *glycines* was determined using nested PCR for 47 soil samples collected from soybean fields in 20 counties of Illinois in 1999. *F. solani* f. sp. *glycines* was detected in soil samples from all five Illinois Agricultural Statistic Districts including 100, 89, 50, 92 and 50% of the samples from East, Central, North-east and West Districts, respectively.

Keywords: detection, *Fusarium solani* f. sp. *glycines*, PCR, soybean, sudden death syndrome

Introduction

Many important fungal pathogens reside in the soil and infect roots of crops, often causing significant yield losses. Sudden death syndrome (SDS) is an economically important soybean disease caused by the soilborne pathogen *Fusarium solani* f. sp. *glycines* (Roy *et al*., 1989; Rupe, 1989) that occurs in most soybean-producing states in the USA (Rupe & Hartman, 1999). In some locations and years, such as in east-central Illinois in 1993, SDS was found in 46% of the soybean fields surveyed, with losses ranging from 20–46% (Hartman *et al*., 1995). SDS is a mid- to late-season disease and symptoms include root rot, crown necrosis, and vascular discoloration of roots and stems. The most conspicuous symptoms of SDS occur on leaves, beginning with chlorotic mottling and proceeding to interveinal chlorosis, necrosis and defoliation. In some years leaf symptoms are less frequent but roots may still be infected. The fungus has been isolated from roots and lower stems, but not from leaves (Rupe, 1989; Roy, 1997). Toxic culture filtrate produced by the fungus has been reported to cause leaf symptoms (Jin *et al*., 1996; Li *et al*., 1999).

Traditional methods to detect or isolate this pathogen include plating plant roots on modified Nash and Snyder's medium (Huang & Hartman, 1996; Cho *et al*., 2001). However, this method is limited by its lack of sensitivity and specificity, as other fungal pathogens with similar morphology to *F. solani* f. sp. *glycines* also grow on these semiselective media (Cho *et al*., 2001). Microscopic methods (Roy, 1997; Li *et al*., 1998) are used to identify *F. solani* f. sp. *glycines*, but it is often difficult to detect the fungus in roots and soil that contain other morphologically similar fungal pathogens. In culture a blue pigmentation of the colony is one characteristic of *F. solani* f. sp. *glycines*, but the colour range varies from light to dark, and some isolates do not produce the blue pigmentation (Rupe *et al*., 1996; Roy, 1997). In addition, other *F. solani* isolates that do not cause SDS also produce blue pigmentation in culture (Nelson & Hansen, 1997).

A molecular approach using random amplified polymorphic DNA (RAPD) markers as a diagnostic tool for the identification of *F. solani* f. sp. *glycines* cultures has been reported (Achenbach *et al*., 1996). O'Donnell & Gray (1995) designed *F. solani* f. sp. *phaseoli*-specific PCR primers based on rDNA sequences and used these primers

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to detect the fungus on inoculated soybean roots. In a previous study, a pair of *F. solani* f. sp. *glycines* primers Fsg1 and Fsg2 were developed based on the mitochondrial small subunit rDNA sequences (Li *et al.*, 2000). At present, reliable and rapid molecular methods to detect this pathogen in field-grown soybeans and soil have not been reported. The objectives of this study were (i) to develop simple and efficient methods to extract fungal DNA directly from plant tissues or soil for use with PCR; (ii) to develop PCR-based methods for the specific detection of *F. solani* f. sp. *glycines* in soybean roots and soil; and (iii) to apply a PCR-based method to evaluate the occurrence of *F. solani* f. sp. *glycines* in soybean fields.

Materials and methods

Fungal cultures

The species, host affiliation, and number of isolates used for PCR amplification and partial sequencing of the mitochondrial small-subunit ribosomal DNA (mtSSU rDNA) and translation elongation factor 1-α gene, and specificity testing of *F. solani* f. sp. *glycines* primers, are listed in Table 1. In addition to 82 *F. solani* f. sp. *glycines* isolates, 55 *F. solani* non-SDS-causing isolates from nine hosts, and 43 other fungi and oomycetes isolated from soybean were tested. All isolates were maintained on 2% water agar (w/v) at 4°C.

Detection assay and plant and soil sample collection and preparation

To develop the detection assay from roots, plants were grown in a growth chamber as previously described (Li *et al.*, 2000). Soybean seeds of a susceptible cultivar, Great Lakes 3202, were sown in Cone-Tainers (Pay Leach Cone-Tainers, Stuewe & Sons, Inc., Corvallis, OR, USA) and inoculated with fungus-infested sorghum grains. Three cm^3 of infested sorghum grains was placed 2–3 cm below a soybean seed in each Cone-Tainer. Non-infested

Table 1 Isolates used for PCR amplification and specificity test of *Fusarium solani* f. sp. *glycines* primers

Fungus/oomycete	Host/substrate	Number of isolates
Fusarium solani f. sp. *glycines*	*Glycine max*	82
F. solani[b]	*G. max*	21
	Phaseolus vulgaris	8
	Pisum sativum	13
	Medicago sativum	3
	Lycopersicum esculentum	2
	Cucurbita pepo	1
	Cucurbita sp.	3
	Solanum tuberosum	2
	Lupinus sp.	1
	Field soil	1
Other fungi isolated from soybean[c]		
Alternaria sp.		1
Cercospora kikuchii		1
Colletotrichum truncatum		1
Diaporthe phaseolorum var. *caulivora*		2
D. phaseolorum var. *meridionalis*		3
D. phaseolorum var. *sojae*		2
F. graminearum		5
F. oxysporum f. sp. *glycines*		1
Fusarium spp.		2
Macrophomina phaseolina		3
Neocosmospora vasinfecta		1
Phomopsis longicolla		5
Phomopsis sp.		1
Phytophthora sojae[d]		5
Phialophora gregata		2
Rhizoctonia solani		2
Septoria glycines		1
Sclerotinia sclerotiorum		4
Stachybotrys chartarum		1
Total isolates		180

[a]Cultures were obtained or isolated from seven States (Arkansas, Illinois, Indiana, Iowa, Kansas, Missouri and Wisconsin) and Argentina.
[b]See Li *et al.* (2000) for more information about these isolates.
[c]All isolates were from Illinois except *F. oxysporum* f. sp. *glycines* (NRRL 22598) and *N. vasinfecta* (NRRL 22166) which were obtained from the Northern Regional Research Laboratory (NRRL), Peoria, IL, USA.
[d]Oomycete.

sorghum grains were used as controls. The Cone-Tainers were placed in a growth chamber with a 16 h photoperiod (300 μM^{-2} s^{-1}) at 28°C day and 22°C night temperature. Plants were tested from 1–4 weeks after sowing. Roots of each plant were removed and washed under running water to remove any adhering soil. After washing, roots were kept in a plastic bag and stored at 4°C until use for plating or kept at –20°C for DNA isolation.

To develop the PCR detection assay from soil, the concentration of macroconidia suspension prepared from a *F. solani* f. sp. *glycines* isolate Mont-1 was determined using a haemocytometer. The macroconidial suspension was then diluted to 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10, before adding 100 μL of each suspension to 1 g twice-autoclaved soil.

Field-grown soybean roots and soil samples were collected from 20 counties in five Agricultural Statistics Districts in Illinois in 1999. Fourteen SDS field-infected plants were left standing in one field in Champaign County in the fall of 1999, and the roots were collected in March 2000. All field soil and root samples were kept in plastic bags and stored at 4°C until use.

In addition, 74 root samples collected from soybean fields in 20 counties of Illinois, cut into 10–15 mm pieces, were surface-disinfested in 1% NaOCl solution for 5 min, rinsed three times in sterile distilled water for 3 min, and plated on a modified Nash and Snyder's medium, a semi-selective medium for *F. solani* f. sp. *glycines* (Huang & Hartman, 1996). Twenty isolates that appeared to be *F. solani* f. sp. *glycines* from plating these root samples were purified and inoculated to soybean plants as described previously (Li *et al*., 2000) to test if they caused SDS.

DNA extraction

Total genomic DNA from pure fungal cultures was extracted from cultures grown on either agar plates or lyophilized mycelium, as described previously (Lee & Taylor, 1990; Li *et al*., 1996; Li *et al*., 2000). Total genomic DNA from inoculated and noninoculated plant roots was isolated with a modified hexadecyltrimethylammonium bromide (CTAB) DNA extraction protocol (Kisha *et al*., 1997). The extraction buffer contained 100 mM Tris-HCl (pH 8·0), 200 mM EDTA (pH 8·0), 1·4 M NaCl, 2% CTAB (w/v), and 1·0% β-mercaptoethanol (v/v).

DNA was extracted from soil samples directly by a modification of two methods (Picard *et al*., 1992; Volossiouk *et al*., 1995). Soil samples (0·25 g per sample) were first lyophilized with liquid nitrogen. A TENP extraction buffer (Picard *et al*., 1992) containing 50 mM Tris (pH 8·0), 20 mM EDTA disodium salt (pH 8·0), 100 mM NaCl and 1% polyvinylpolypyrrolidone (Sigma, St Louis, MO, USA) was added to the lyophilized soil suspended in 0·5 mL 0·4% dry milk powder solution (Volossiouk *et al*., 1995). After vortexing, samples were incubated at 65°C for 30 min, then 10 μL of 20% (w/v) sodium dodecyl sulfate (SDS) was added to the sample to make a final concentration of 1%. The samples were incubated at 65°C for another 30 min. In addition, a TENS buffer (Kuske *et al*., 1998) containing 50 mM Tris (pH 8·0), 20 mM EDTA, 100 mM NaCl, 1% (w/v) sodium dodecyl sulfate, plus 10 μg mL^{-1} proteinase K was also used as DNA extraction buffer (incubation temperature was 55°C for 1 h) for comparison. After incubation, samples were centrifuged at 18 000 *g* for 10 min to remove soil and debris. The supernatant was transferred to a new centrifuge tube. DNA was precipitated with half volume of 7·5 M ammonium acetate and two volumes of 100% ethanol at –20°C for 2 h or overnight. DNA was pelleted, rinsed with 70% ethanol, dried, dissolved in sterile water or 0·1 × TE buffer (10 mM Tris–Cl pH 8·0 and 1 mM EDTA pH 8·0) and used as a template for PCR amplification or stored at –20°C until use.

PCR amplification

PCR amplification of the mtSSU rDNA region was performed using fungal primer set NMS1/NMS2 (Li *et al*., 1994) and the translation elongation factor 1-α gene primer set EF1-728F/EF1-986R (Carbone & Kohn, 1999). PCR was performed in either a 15 μL (for general sample test) or 100 μL (for DNA sequencing and PCR sensitivity tests) mixture that contained 0·2 mM each of dATP, dCTP, dGTP and dTTP, 50 mM KCl, 20 mM Tris–HCl pH 8·4, 2·0 mM $MgCl_2$, 0·2 μM of the primers, 25 ng and 100 ng genomic DNA for the 15 and 100 μL reaction, respectively, and 0·5 U (for 15 μL reaction) or 2·5 U (for 100 μL reaction) *Taq* DNA polymerase (Gibco BRL Life Technologies, Gaithersberg, MD, USA). For DNA extracted from soil or roots, 0·1% (w/v) bovine serum albumin (Sigma) was added in the PCR mixture. Negative controls that excluded the DNA template, or 'DNA' isolated from autoclaved soil were included in every experiment to test for reagent contamination. Amplification was performed with a GeneAmp PCR System 2400 DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, CA, USA) programmed for one cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 60 s, and 72°C for 90 s. A 10 min extension at 72°C was conducted after 30 cycles. Detection of *F. solani* f. sp. *glycines* from plant and soil samples used a 'touch-down' PCR approach (Don *et al*., 1991) to ensure specificity of product amplification. This consisted of one cycle at 94°C for 5 min, followed by 10 cycles of 94°C for 30 s, 66°C for 60 s with a 1°C decrease in every cycle, and 72°C for 90 s, and then 20 cycles at 94°C for 30 s, 56°C for 60 s, 72°C for 90 s with a 7 min extension at 72°C on the final cycle. Sensitivity of PCR was tested using different concentrations of *F. solani* f. sp. *glycines* DNA (isolate Mont-1) at 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per microlitre in a 100 μL PCR reaction. Detection of *F. solani* f. sp. *glycines* in field-grown soybean roots and soil samples used a nested PCR approach that included two rounds of amplification using universal primers NMS1 and NMS2, or EF-728F and EF-986R for the first round and the internal *F. solani* f. sp. *glycines*-specific primers Fsg1 and Fsg2, or FsgEF1 and FsgEF2 for the second. PCR products were examined by electrophoresis of 5 μL

aliquots on a 1·5 or 2% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator (Fotodyne Inc., Hartland, WI, USA).

DNA sequencing

Forty isolates, including 16 *F. solani* f. sp. *glycines* and 24 other *F. solani*, were sequenced for the NMS1 and NMS2 amplified mtSSU rDNA region (Li *et al*., 2000). Thirty isolates including 13 *F. solani* f. sp. *glycines* and 17 other soybean pathogens were sequenced for a portion of the translation elongation factor 1-α gene. Prior to sequencing, PCR products were purified by filtration though Ultrafree-MC low protein-binding regenerated cellulose membrane filter units (NMWL) (Millipore, Bedford, MA, USA) according to the manufacturer's instructions.

Approximately 30–50 ng of the purified PCR products were subjected to sequencing using the Dye Terminator Cycle Sequencing Kit with an Applied Biosystem 373 A sequencer (Perkin Elmer, Foster City, CA, USA). The conditions for the cycle-sequencing reactions were: 1 min at 95°C for initial denaturation, followed by 25 cycles of 15 s at 95°C, 5 s at 45°C, and 4 min at 60°C. Both DNA strands were sequenced with each primer set, NMS1/NMS2 and EF1-728F/EF1-986R.

Sequence data analyses and primer design

DNA sequences were edited using Sequencher (Gene Codes Corp., Ann Arbor, MI, USA) and aligned using the multiple sequences alignment program CLUSTAL W (Thompson *et al*., 1994). To design specific PCR primers to *F. solani* f. sp. *glycines*, sequence data from the present and previous (Li *et al*., 2000) studies were analysed and compared with fungal sequences in GenBank. The GenBank accession numbers used for the analysis of small-subunit mitochondrial rRNA genes were from AF124995 to AF125008, and from AF125009 to AF125032; for the translation elongation 1-α gene the GenBank accession numbers were from AF395645 to AF395657, from AY04380 to AY04382, and from AF398887 to AF398900. Potential primer sequences were tested for performance characteristics such as hairpin structure, potential self-dimer formation, and stability of 3′ termini by using the PRIMER 3 software package (S. Rozen and H. J. Skaletsky, Whitehead Institute for Biomedical Research, Cambridge, MA, USA).

Results

Specificity and sensitivity of the PCR amplification

All isolates studied gave strong amplification of a single PCR product of about 600 bp using primers NMS1/NMS2 (Fig. 1a) and of 320 bp using primers EF-728F/EF-986R (Fig. 3a). One set of specific primers for *F. solani* f. sp. *glycines*, Fsg1/Fsg2, designed from the mtSSU rDNA region, had the following sequences: 5′-GTC TTC TAG GAT GGG CTG GT-3′ and 5′-CAT TTA ATG CCT AGT CCC CTA TCA-3′, respectively. The other set of *F. solani* f. sp. *glycines*-specific primers, FsgEF1/FsgEF2, designed from the translation elongation factor 1-α gene, had the following sequences: 5′-GAGTCGGTTAGCTTCTGTC-3′ and 5′-GCGCGCCTTGCTATTCTCC-3′, respectively. These two sets of *F. solani* f. sp. *glycines*-specific primers produced PCR products of 438 (Fig. 2b) and 237 bp (Fig. 3b), respectively. All 82 *F. solani* f. sp. *glycines* isolates from seven states in the USA and Argentina had single amplified PCR products using *F. solani* f. sp. *glycines*-specific primers. No amplification occurred when the specific primers were tested with 55 *F. solani* non-SDS isolates, 38 other fungal isolates and five oomycete isolates from soybean, host soybean and autoclaved soil. All fungal and oomycete isolates tested had a positive PCR reaction using the fungal universal primers NMS1/NMS2 and EF728F/EF986R.

Sensitivity of the primer set Fsg1/Fsg2 was 10 pg (Fig. 2b), and for FsgEF1/FsgEF2 was 1 ng (Fig. 3b) from

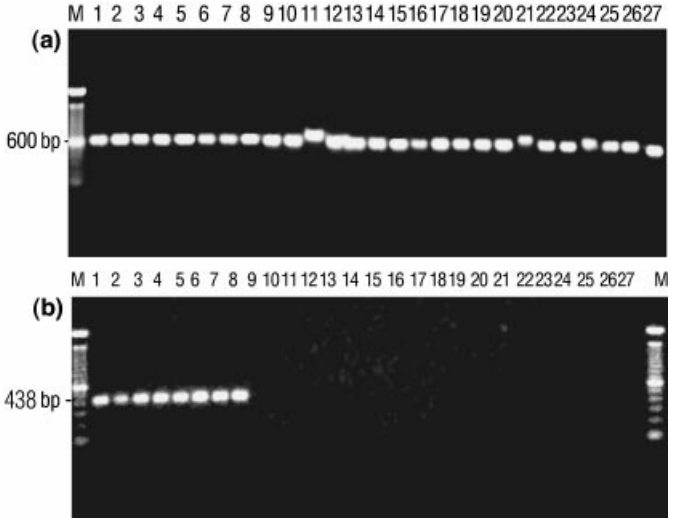

Figure 1 Gel electrophoresis of PCR-amplified products using (a) primers NMS1/NMS2 and (b) *Fusarium solani* f. sp. *glycines* (FSG)-specific primers Fsg1/Fsg2. Lane M, 100 bp DNA ladder; lanes 1–8, FSG isolates; lanes 9–18, *F. solani* non-sudden death syndrome-causing isolates; lanes 19–27, other fungal isolates from soybean.

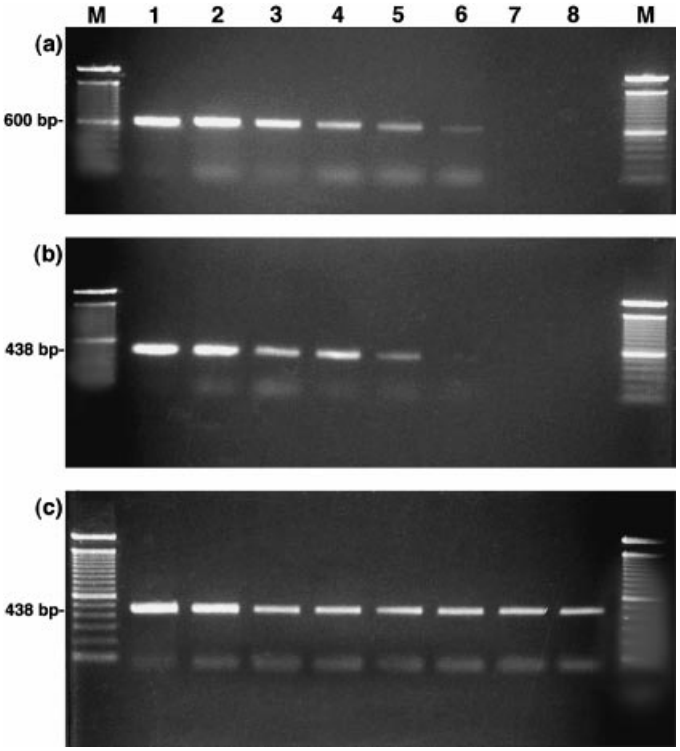

Figure 2 Sensitivity of PCR with (a) primers NMS1/NMS2 and (b) Fsg1/Fsg2 using different concentrations of *Fusarium solani* f. sp. *glycines* (FSG) DNA; and (c) nested PCR using primers NMS1/NMS2 for the first round of amplification and primers Fsg1/Fsg2 for the second amplification. Lane M: 100 bp DNA ladder; lanes 1–8, FSG DNA at concentrations of 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg μL^{-1} in a 100 μL PCR reaction.

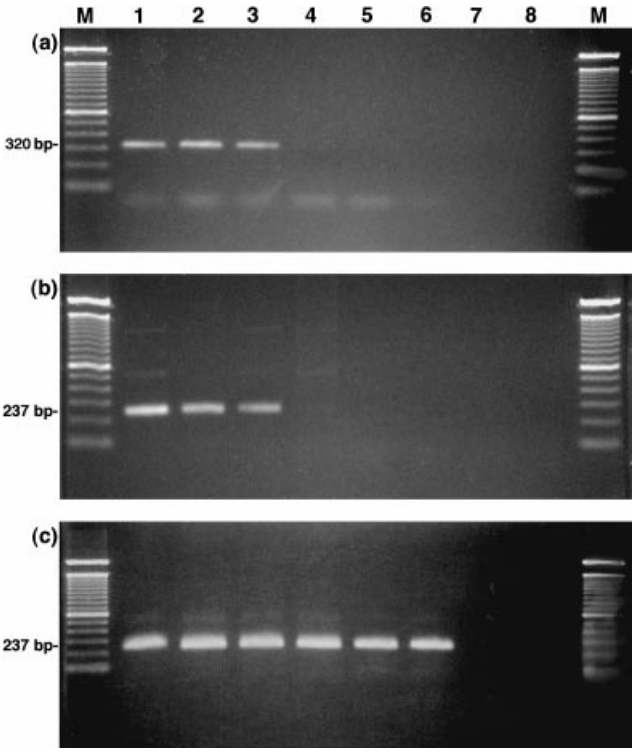

Figure 3 Sensitivity of PCR with (a) primers EF1-728F/EF1-986R and (b) FsgEF1/FsgEF2 using different concentrations of *Fusarium solani* f. sp. *glycines* (FSG) DNA; and (c) nested PCR using primers EF1-728F/EF1-986R for the first round of amplification and primers FsgEF1/FsgEF2 for the second amplification. Lane M, 100 bp DNA ladder; lanes 1–8, FSG DNA at concentrations of 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 1 fg μL^{-1} in a 100 μL PCR reaction.

pure template of *F. solani* f. sp. *glycines* total genomic DNA and down to 10^3 macroconidia g^{-1} soil (Fig. 4). Nested PCR increased the sensitivity of primers 1000-fold to 10 fg for primers Fsg1/Fsg2 (Fig. 2c) and 1 pg (Fig. 3c) for primers FsgEF1/FsgEF2.

Detection of *F. solani* f. sp. *glycines* in soybean roots

A 438 bp PCR amplification product was obtained using primers Fsg1 and Fsg2 on all inoculated soybean roots (Table 2). Roots of inoculated symptomless seedlings

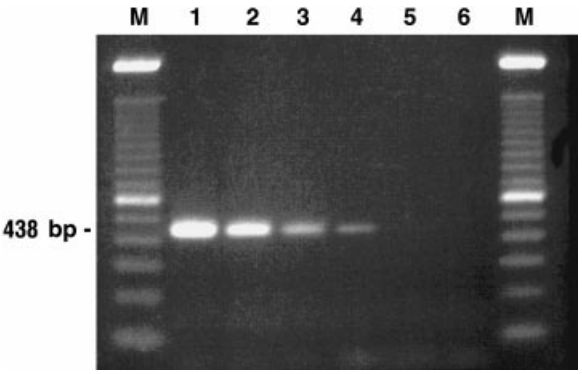

Figure 4 Sensitivity of PCR with *Fusarium solani* f. sp. *glycines* (FSG) primers Fsg1 and Fsg2 using soils containing different concentrations of FSG macroconidia. Lane M, 100 bp DNA ladder; lanes 1–6, number of FSG macroconidia g^{-1} soil was 10^6, 10^5, 10^4, 10^3,10^2 and 10, respectively.

were positive for PCR amplification 7 days after inoculation. PCR amplification occurred on inoculated soybean roots using either specific primers Fsg1 and Fsg (Fig. 5a) or a combination of two sets of primers Fsg1/Fsg2 and FsgEF1/FsgEF2 (Fig. 5b). *F. solani* f. sp. *glycines* was detected in nine of 14 (65%) field-grown soybean roots from SDS-diseased plants that were left in the field over the winter and collected in March 2000 using primers Fsg1/Fsg2 (Table 2; Fig. 6) and FsgEF1/FsgEF2 (Table 2). In addition, 64% of soybean roots collected in August 1999 from soybean fields in 20 Illinois counties produced 438 bp products in the specific PCR assay using primers Fsg1/Fsg2 (Table 3; Fig. 6) and 237 bp amplification products using FsgEF1/FsgEF2 (Table 3). Two randomly picked root samples that had PCR products amplified by Fsg1/Fsg2, and two amplified by FsgEF1/FsgEF2, had 100% identical DNA sequence with all previously sequenced *F. solani* f. sp. *glycine* isolates (Li *et al.*, 2000 and this study). This confirmed that the Fsg1/Fsg2- and

Table 2 Detection of *Fusarium solani* f. sp. *glycines* (FSG) in soybean roots by direct DNA extraction and PCR using primer sets NMS1/NMS2 and Fsg1/Fsg2

Root samples	Foliar disease severity[a]	Days after planting	No. roots tested	Percentage positive using PCR	
				NMS1/NMS2	Fsg1/Fsg2
Noninoculated[b]	1	7–12	10	0	0
Inoculated[b]	1	7	8	100	100
Inoculated[b]	2–3	10–14	10	100	100
Inoculated[b]	4–5	14–28	22	100	100
Soybean field[c]	3–5	≈100	20	85	65
Soybean field[d]	3–5	≈300	14	86	64

[a]1 = No foliar symptoms to 5 = most severe foliar symptoms (Hartman *et al.*, 1997).
[b]Plants were grown in an equal volume of a sand : soil mix, twice-autoclaved, and inoculated when seeds were sown.
[c]Soybean roots collected in August 1999 from soybean fields in 20 counties in Illinois.
[d]Roots of 1999 infected plants overwintered in the field and collected in March 2000. The same DNA from root samples was also tested with PCR using primer set EF-786F/EF-986 and FsgEF1/FsgEF2, results were the same as using primers sets NMS1/NMS2 and Fsg1/Fsg2.

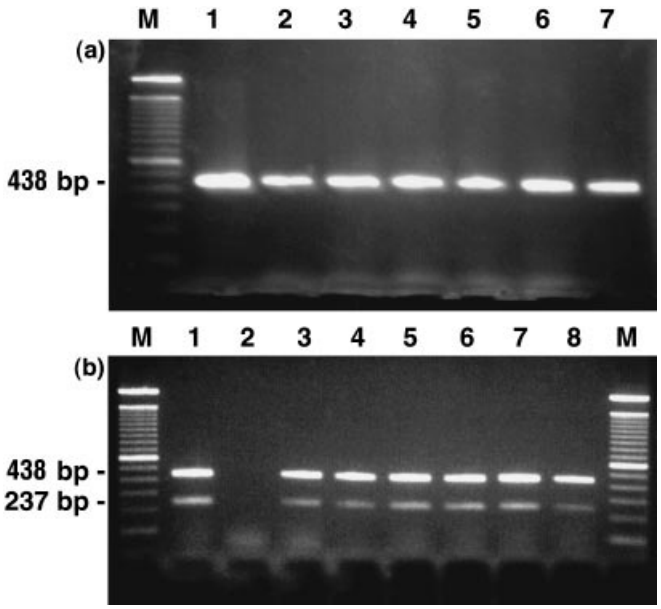

Figure 5 Specific PCR of inoculated soybean roots using (a) *Fusarium solani* f. sp. *glycines* (FSG)-specific primers Fsg1/Fsg2 and (b) a combination of two sets of FSG-specific primers Fsg1/Fsg2 and FsgEF1/FsgEF2. Lane M, 100 bp DNA ladder. (a) Lane 1, FSG DNA; lanes 2–7, roots obtained from inoculated soybean plants grown in a growth chamber; (b) lane 1, FSG DNA; lane 2, soybean root DNA; lanes 3–8, roots obtained from inoculated soybean plants grown in a growth chamber.

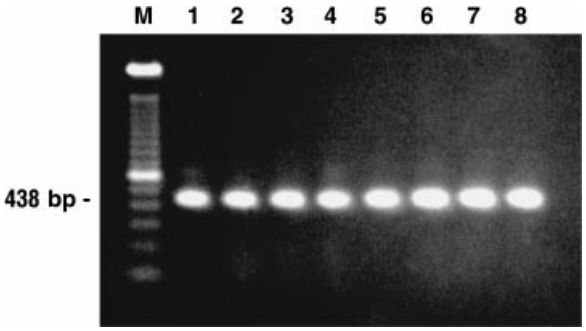

Figure 6 Nested PCR tested on field-grown soybean root samples that included two rounds of amplification using universal primers NMS1/NMS2 for the first round and the internal *Fusarium solani* f. sp. *glycines* (FSG)-specific primers Fsg1/Fsg2 for the second. Lane M, 100 bp DNA ladder; lanes 1–4, field soybean roots from sudden death syndrome (SDS) diseased plants collected in September 1999; lanes 5–8, field soybean roots from SDS plants that overwintered in the field and were collected in March 2000.

Table 3 Detection of *Fusarium solani* f. sp. *glycines* by direct DNA extraction and a nested PCR using primer sets NMS1/NMS2 (first round) and Fsg1/Fsg2 (second round) in soil and root samples collected from soybean fields in five Illinois Agricultural Statistics Districts

	Soil samples[a]		Root samples[a]	
District	No.	PCR (%)	No.	PCR (%)
East[b]	10	100	13	85
Central[b]	9	89	6	83
North-east	8	50	–[c]	–[c]
North-west	12	92	1	100
West	8	50	–[c]	–[c]

[a]Each sample was collected from a different county in three fields (three subsamples) in 1999, except three extra samples were taken from Champaign county in the East District.
[b]The same DNA of the samples from soil and roots were also tested with a nested PCR using primer set EF-786F/EF-986 (first round) and FsgEF1/FsgEF2 (second round). Results were the same as using primers sets NMS1/NMS2 (first round) and Fsg1/Fsg2 (second round).
[c]Not sampled.

FsgEF1/FsgEF2-amplified PCR products in root DNA were from *F. solani* f. sp. *glycine* isolates.

In addition, 49 colonies from plating of field soybean root samples that appeared similar in cultural morphology to purified isolates of cultures of *F. solani* f. sp. *glycines* were selected from a modified Nash and Snyder's medium (Huang & Hartman, 1996). Results of pathogenicity test on 20 isolates showed that six (30%) did not cause SDS foliar symptoms, although they still caused root rot of soybean. Some of these isolates produced microconidia and were identified as other *Fusarium* species. Results of PCR assay using specific primers Fsg1/Fsg2 also were negative for these six isolates.

Detection of *F. solani* f. sp. *glycines* in field soil

DNA extracted from 47 field soil samples from 20 counties in Illinois was subjected to the PCR using universal fungal primers NMS1 and NMS2. Direct detection of *F. solani* f. sp. *glycines* failed in almost all (>95%) field soil samples using regular PCR, as PCR products were very weak or not detectable. Using a nested PCR approach with primers NMS1/NMS2 for the first round, and the specific primers Fsg1/Fsg2 for the second amplification, *F. solani* f. sp. *glycines* was detected in soil samples from all five Illinois Agricultural Statistic Districts including 100, 89, 50, 92 and 50% of the samples from East, Central, North-east, North-west, and West Districts, respectively (Table 3). Another nested PCR using primers EF-728F/EF-986R for the first round and FsgEF1/FsgEF2 for the second also successfully detected *F. solani* f. sp. *glycines*, and the PCR product was 237 bp (data not shown). Two randomly picked soil samples that had 438 bp PCR products amplified by Fsg1/Fsg2 had 100% identical DNA sequences with previously published DNA sequences of 14 *F. solani* f. sp. *glycines* isolates (Li *et al*., 2000). Two randomly picked soil samples that had 237 bp PCR products amplified by FsgEF1/FsgEF2 also had 100% identical DNA sequences with DNA sequences of 13 *F. solani* f. sp. *glycines* isolates in this study. This confirmed that the Fsg1/Fsg2- and FsgEF1/FsgEF2-amplified PCR products in soil DNA were from *F. solani* f. sp. *glycines* isolates.

Discussion

PCR-based assays have been applied to microbial ecology and environmental sciences to detect and monitor microorganisms in rhizosphere/rhizoplane and soils (Steffan & Atlas, 1991), and to plant disease diagnosis (Henson & French, 1993). Field-grown plant roots and field soils are microbially complex, making it difficult to develop convenient and reliable procedures for extracting PCR-amplifiable DNA (Steffan & Atlas, 1991; Lovic *et al*., 1995). Extraction of DNA from soil samples often results in coextraction of humic substances that interfere with DNA detection (Zhou *et al*., 1996; Wilson, 1997). This contamination can inhibit *Taq* DNA polymerase activity in PCR (Tsai & Olson, 1992; Smalla *et al*., 1993). One of the objectives of the present study was to develop simple and effective methods to extract *F. solani* f. sp. *glycines* DNA directly from field-grown roots and soils. CTAB and PVPP were used to remove humic contamination from extracted DNA. Although neither completely removes humic compounds, both were shown significantly to decrease the concentration of contaminants (Zhou *et al*., 1996). For direct DNA extraction from soil, a TENP extraction buffer was used. This was originally developed to isolate bacterial DNA in soil because it has EDTA that protects the DNA from nuclease activity, sodium chloride as a dispersing solution, and the insoluble polyvinylpolypyrrolidone that removes humic acids and other phenolic impurities (Picard *et al*., 1992). When the TENP method was used to isolate fungal DNA from soil, only

six out of 20 DNA samples were PCR positive using fungal universal primers NMS1 and NMS2. However, when sodium dodecyl sulfate was added to TENP-treated soil samples, an increase of positive PCR amplification occurred in all samples tested. This method is referred to as the TENP + SDS (sodium dodecyl sulfate) DNA soil isolation method. In addition, adding 0·4% dry milk solution to soil samples before extraction (Volossiouk *et al.*, 1995) also helped to block the background and increased the frequency with which PCR-amplifiable DNA was obtained from soil. Without this step, DNA isolation from field soil was less successful.

The TENP + SDS DNA isolation method developed was successful for the soil types used in this study, and it would be interesting to determine if this method was successful over a wide range of soil types. Another method, TENS + proteinase K, which is similar to a recently reported method to detect and quantify *Verticillium tricorpus* propagules in soil (Heinz & Platt, 2000), also provided PCR-amplifiable DNA from soil, but was not as efficient as the TENP + SDS method because proteinase K is temperature-sensitive. Development of a direct DNA extraction method from soil or plant tissues without the need of isolating and obtaining pure cultures of the pathogen is an important step in developing a quick PCR-based detection assay. In addition, this method may be useful for detecting many microorganisms that cannot be cultured.

PCR sensitivity is a concern in any pathogen-detection method using field samples. One approach to increasing sensitivity is to use nested PCR. In this study, nested PCR includes two rounds of amplification using universal primers for the first round to increase the target DNA templates, then using the internal specific primers for the second round. Detection of *F. solani* f. sp. *glycines* in field soil samples using a single round of PCR was not consistent, probably because of the low concentration of the target DNA. By contrast, the nested PCR approach presented here produced consistent and reproducible results. Nested PCR has been reported to increase PCR sensitivity by 10–1000 times (McManus & Jones, 1995; Faggian *et al.*, 2000; Judelson & Tooley, 2000). In the present experiments, nested PCR increased the sensitivity of *F. solani* f. sp. *glycines*-specific primers by 1000 fold to 10 fg. The sensitivity of the nested PCR assay using primers Fsg1/Fsg2 was 100 times higher than the FsgEF1/FsgEF2 nested PCR assay. Also, the size of the PCR product amplified by Fsg1/Fsg2 (438 bp) was greater than that amplified by FsgEF1/FsgEF2 (237 bp), which allowed it to be detected more easily. The nested PCR assay has potential as a diagnostic tool for detecting *F. solani* f. sp. *glycines* in field-grown soybean roots and soil. It also may be useful for detection of *F. solani* f. sp. *glycines* in seed lots and debris associated with seed lots.

Another objective was to use the methodology developed for DNA extraction and PCR assay on the specific detection of *F. solani* f. sp. *glycines* in plants and soil samples. Detection of *F. solani* f. sp. *glycines* using the described PCR assay was positive for all SDS-diseased plants expressing symptoms, infected but symptomless young seedlings and infested field soils. However, *F. solani* f. sp. *glycines* could not be isolated from all SDS-infected roots or soil that was positive in the PCR assay. This may be, in part, because of the difficulty of isolating *F. solani* f. sp. *glycines*, as it grows slowly compared to aggressive contaminants when present in the sample. It may also be due to the low frequency of *F. solani* f. sp. *glycines* propagules compared to the high frequency of other aggressive microorganisms in roots or soil. Also, infected roots or soil with nonviable *F. solani* f. sp. *glycines* might test positive with PCR even though the fungus cannot be isolated, as DNA can be amplified from dead mycelium (Henson & French, 1993) if the template part of the DNA has not completely degraded. Recently, *Phytophthora infestans* mitochondrial DNA (mtDNA) from 19th century herbarium specimens was PCR-amplified and sequenced to unravel the genealogical history of the potato late blight pathogen and to identify the mtDNA lineage(s) involved in 19th century epidemics (Ristaino *et al.*, 2001). The PCR assay developed in this study would be useful to survey the incidence of SDS or the occurrence and distribution of *F. solani* f. sp. *glycines* in soil.

In addition, detection of *F. solani* f. sp. *glycines* using traditional agar plating does not distinguish the *forma specialis* in fields with a history of both *Glycine max* and *Phaseolus vulgaris*, and other *F. solani* non-SDS isolates that have similar morphology to *F. solani* f. sp. *glycines* (Nelson & Hansen, 1997) and also grow well in the currently available semiselective media (Cho *et al.*, 2001). The PCR assay developed in this study would be useful to survey more accurately the occurrence and distribution of *F. solani* f. sp. *glycines* in soil, as well as to provide a positive diagnosis for the occurrence of *F. solani* f. sp. *glycines* in roots.

In this study, DNA was extracted from 47 soil samples collected from different soybean production regions in Illinois. *Fusarium solani* f. sp. *glycines* was detected in all soil samples from eastern Illinois, and in 89, 50, 92 and 50% of the samples from central, north-eastern, north-western, and western Illinois, respectively. Further experiments using more samples are in progress to determine more accurately the occurrence and distribution of *F. solani* f. sp. *glycines* in Illinois. In addition, the development of a quantatitive PCR-based assay for *F. solani* f. sp. *glycines* in field soil and in different soybean genotypes will provide more detail about the population levels that occur in soil and plants. Using the Smart-Cycler System (Gepheid, Sunnyvale, CA, USA) with fluorescence-labelled primers or intercalating dyes in the PCR amplification has allowed a standard curve of PCR product quantities to be constructed from known concentrations of *F. solani* f. sp. *glycines* DNA (S. Li, unpublished data). Quantification of *F. solani* f. sp. *glycines* DNA in soil and soybean root samples from different locations of Illinois using this PCR-based assay is in progress.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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